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Effects of Salts on the Halophilic Alga *Dunaliella viridis*[1]

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Determinations of the salt sensitivity of enzymes extracted from the halophilic alga *Dunaliella viridis* revealed that pentose phosphate isomerase, ribulose diphosphate carboxylase, glucose-6-phosphate dehydrogenase, and phosphohexose isomerase were inhibited by NaCl concentrations far lower than that in the growth medium (3.75 M). The inhibition was reversible and was not prevented by preparing the extracts in the presence of salt. Potassium, lithium, and cesium chlorides were equally inhibitory. In contrast, whole cells require rather high levels of NaCl for optimal growth, whereas growth is inhibited by low levels of the other cations. The results suggest a specific mechanism for the exclusion of sodium from the interior of the cell.

The basis of the salt tolerance of the halophilic bacteria appears to be a distinctive form of enzymatic protein which is resistant to inactivation by high levels of salt and, in fact, requires substantial concentrations of salt for full enzymatic activity (6). The halophilic species of the green alga *Dunaliella* grow in concentrated salt solutions, but the basis of this salt tolerance is unknown. The present report describes the effects of sodium chloride and other salts on the growth of *Dunaliella viridis* and on the activity of several enzymes from this halophilic species.

MATERIALS AND METHODS

Isolation of culture. The axenic culture used in these experiments was isolated from a solar evaporation pond in San Francisco Bay by serial passage of the sample through the mineral medium containing 3.75 M NaCl (supplemented with penicillin and streptomycin to prevent bacterial growth). A pure culture, free from bacteria (as determined by plating on nutrient agar containing various levels of salt), was obtained by picking an isolated clone on solid medium.

The genus *Dunaliella* was first described by Teodoresco (17, 18). He described two species, *D. salina* and *D. viridis*, differing mainly in that the former becomes red in media of high salt concentration, whereas the latter remains green under all conditions. Lerche (7) recommended abandonment of the species *D. viridis* and described a number of new species of *Dunaliella*. The designation "viridis" has, however, continued in the literature and seems best to describe the culture used in these experiments, since our cultures appeared green at all salt concentrations tested.

The cells used in these experiments are illustrated in the photomicrograph (Fig. 1). The organisms measure approximately 12×7 μ and contain a cup-shaped chloroplast, two anterior flagellae, and a red "eye-spot."

Growth of cultures. D. viridis was grown in a synthetic medium containing the following components (per liter): $MgCl_2 \cdot 6H_2O$, 1.5 g; $MgSo_4 \cdot 7H_2O$, 0.5 g; KCl, 0.2 g; $CaCl_2$, 0.2 g; KNO_3, 1.0 g; $NaHCO_3$, 0.043 g; tris(hydroxymethyl)aminomethane (Tris), 2.45 g (*p*H adjusted to 7.5 with HCl); KH_2PO_4, 0.035 g; ethylenediaminetetraacetate, 1.89 mg; $FeCl_3 \cdot 6H_2O$, 2.44 mg; $ZnCl_2$, 0.041 mg; H_3BO_3, 0.61 mg; $CoCl_2 \cdot 6H_2O$, 0.015 mg; $CuCl_2 \cdot 2H_2O$, 0.041 mg; $MnCl_2 \cdot 4H_2O$, 0.41 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.38 mg; and $VOCl_2$, 0.041 mg. The level of sodium chloride in the medium is indicated with each experiment. The potassium phosphate solution was autoclaved separately and added aseptically to the sterile medium. The cultures were grown in 2,000-ml Erlenmeyer flasks containing 500 ml of medium and were incubated on a rotary shaker at 25 C for 7 to 10 days

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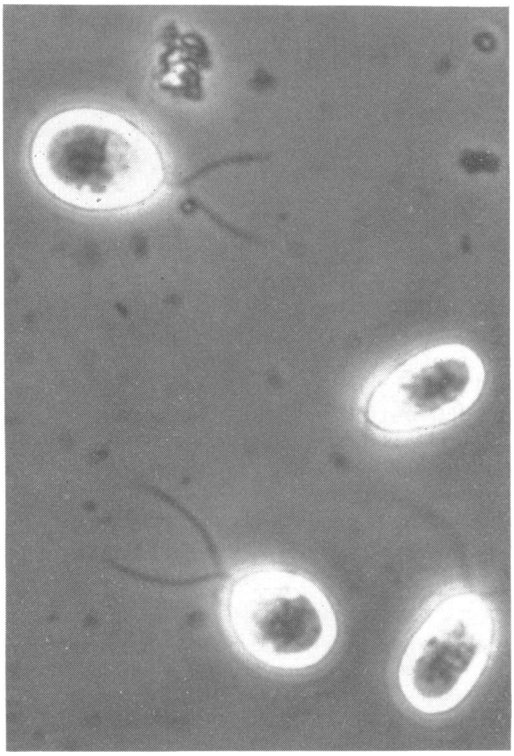

FIG. 1. *Dunaliella viridis. Cells grown in medium containing 3.75 M NaCl. Magnification × 1,355; phase-contrast optics.*

with a light intensity of approximately 300 ft-c. For growth experiments, the volume of medium was reduced to 250 ml with an initial inoculum of 25,000 cells per ml. Cell counts were made with a hemocytometer on properly diluted or concentrated samples. Two to four samples from each duplicate culture were counted, and each experiment was repeated at least once.

Preparation of extracts. The cells were harvested by centrifugation, then washed once in a sodium chloride solution of the same concentration as that of the growth medium, and suspended (1 to 2 g/5 ml) in 0.05 M Tris-chloride buffer (*p*H 7.5), or, where so indicated, in buffered salt solution. The cells were disrupted in a French press at 20,000 psi, followed by centrifugation at 20,000 × *g* for 30 min. The supernatant fluid is referred to as the crude extract. In some cases, the extract was further clarified by centrifuging at 100,000 × *g* for 20 min. The supernatant fluid from this centrifugation is termed 100_S20. When so indicated, salt was removed from this fraction by dialysis [against 1 liter of 0.05 M Tris-chloride (*p*H 7.5) for 1 hr with three changes of solution during that period].

Extracts of *Thiobacillus thioparus* were prepared by the methods described by Mayeux and Johnson (13) for *T. neapolitanus.*

Enzyme assays. The fixation of carbon dioxide into acid-stable products was measured as described by Johnson and Peck (4). The assay was performed in a total volume of 2.7 ml at 30 C in a helium atmosphere.

Glucose-6-phosphate dehydrogenase activity was assayed spectrophotometrically by following the reduction of nicotinamide adenine dinucleotide triphosphate (NADP) at 340 mμ. The assay mixture (unless otherwise indicated) contained (in μmoles): Tris-chloride or glycylglycine buffer, *p*H 7.5, 250; $MgSO_4$, 10; NADP, 2.6; glucose-6-phosphate (sodium salt), 1.0; cell extract, 0.1 or 0.2 ml. The assay was performed in a total volume of 3.0 ml at room temperature. The results were not corrected for the low levels of 6-phosphogluconate dehydrogenase activity in these extracts.

Phosphoglucose isomerase was assayed by incubation of 5 μmoles of glucose-6-phosphate, 20 μmoles of Tris-chloride buffer (*p*H 8.0), and 0.05 ml of a 1:10 dilution of crude extract in a total volume of 0.6 ml for 15 min at 30 C. Ketose was detected by the method of Roe (16); the reaction was stopped by the addition of 7 ml of 10 M HCl, followed by 1 ml of resorcinol solution (0.1 g of resorcinol and 0.25 g of thiourea per 100 ml of glacial acetic acid). The color was developed by incubation for 10 min at 80 C and, after cooling, the optical density was measured on a Klett-Summerson colorimeter with a no. 54 filter.

Pentose phosphate isomerase was measured by the method of Axelrod and Jang (1). The incubation mixture contained: ribose-5-phosphate (sodium salt), 1.2 μmole; Tris-chloride buffer (*p*H 7.5), 50 μmoles; and diluted crude extract, 0.1 ml (approximately 0.01 mg of protein).

All enzyme assays were performed with a level of enzyme having activity well within the range in which activity was proportional to enzyme concentration. Protein was determined by the method of Lowry et al. (8).

Source of chemicals. Ribulose-1,5-diphosphate was purchased from Sigma Chemical Co., St. Louis, Mo. Other biochemicals were purchased from Sigma Chemical Co. and from Calbiochem, Los Angeles, Calif.

RESULTS AND DISCUSSION

Effect of NaCl on CO_2 fixation. The results of an experiment comparing the sensitivity to inhibition by NaCl of adenosine triphosphate (ATP)-dependent CO_2 fixation in *D. viridis*, a halophilic alga grown in 3.75 M salt, and *Thiobacillus thioparus*, a nonhalophilic autotrophic bacterium, are shown in Table 1. The *D. viridis* system was inhibited significantly at a sodium chloride concentration of 0.076 M; at 0.17 M (1% NaCl), the fixation of CO_2 was inhibited by 56%. In *T. thioparus*, no inhibition was observed at 0.076 M and only slight inhibition (7%) at 0.17 M. It appears, therefore, that the CO_2-fixing system in *D. viridis* is even more sensitive to inhibition by salt than the CO_2-fixing system from the nonhalophilic *T. thioparus*.

TABLE 1. *Effect of NaCl on ATP-dependent $^{14}CO_2$ fixation*[a] *in extracts of Dunaliella viridis*[b] *and Thiobacillus thioparus*

System	Concn of NaCl (M)	CO_2 fixed (μmoles/10 min)	Percentage of inhibition
D. viridis (1 mg of protein)	0	0.27	0
	0.038	0.27	0
	0.076	0.23	15
	0.170	0.12	56
	1.70	0.02	93
T. thioparus (2 mg of protein)	0	2.9	0
	0.038	3.1	0
	0.076	3.0	0
	0.17	2.7	7
	1.70	0	100

[a] Incubation mixture contained (in μmoles): Tris chloride, 100; $MgCl_2$, 60; $Na_2{}^{14}CO_3$, 10 (10^6 counts per min per μmole); ribose-5-phosphate, 5; ATP, 5.

[b] Grown in 3.75 M salt.

Table 2 shows the results of a similar experiment with cells of *D. viridis* grown in a lower salt concentration (1.28 M). Inhibition produced by 0.17 M NaCl was only slightly higher than in the previous case. Clearly, the NaCl tolerance of the CO_2-fixing system was not significantly lessened when the cultures were grown in a lower salt concentration. When ribose-5-phosphate and adenosine triphosphate are replaced by ribulose-1,5-diphosphate in this assay (Table 2), the fixation rates reflect the activity of carboxydismutase. The sensitivity of this enzyme to salt is seen to be similar to that observed when the fixation involves all the carboxylative phase enzymes (phosphoribose isomerase, phosphoribulokinase, and carboxydismutase). The effect on phosphoribose isomerase alone is shown in Table 3. Cells grown in 1.28 and 3.75 M salt showed similar sensitivity to 0.17 M NaCl (23 and 33% inhibition, respectively). As was the case with the other enzyme systems, inhibition was virtually complete at 1.7 M NaCl.

Since the fixation of CO_2 is absolutely necessary for the growth of these cells, the extreme salt sensitivity of the enzymes involved in CO_2 fixation may indicate that they do not normally function at a high salt level and that *D. viridis* is able to maintain an internal salt concentration considerably lower than that of the medium in which it grows. It seemed desirable, however, to have information on the salt sensitivity of other enzymes and to examine various factors which might contribute to an apparent salt sensitivity.

TABLE 2. *Effect of NaCl on ATP-dependent $^{14}CO_2$ fixation*[a] *in extracts of Dunaliella viridis grown in 1.25 M NaCl*

System	Concn (M)	CO_2 fixed (μmole/10 min)	Percentage of inhibition
Ribose-5-phosphate	0	0.443	0
	0.170	0.142	68
	0.425	0.061	86
	0.850	0.018	96
	1.70	0.015	97
Ribulose-1,5-diphosphate	0	0.469	0
	0.170	0.189	60
	0.425	0.117	75
	0.850	0.034	93
	1.70	0.020	96

[a] Incubation mixture contained (in μmoles): Trischloride, 100; $MgCl_2$, 16; $Na_2{}^{14}CO_3$, 30 (250,000 counts per min per μmole); either ribose-5-phosphate, 10, and ATP, 20, or ribulose-1,5-diphosphate, 4; and 0.2 ml of extract containing 1.2 mg of protein.

TABLE 3. *Effect of NaCl on phosphoribose isomerase from Dunaliella viridis*

Concn of NaCl (M)	Grown in 1.28 M NaCl		Grown in 3.75 M NaCl	
	Amount of ribulose-5-phosphate per min per mg of protein (μmoles)	Percentage of inhibition	Amount of ribulose-5-phosphate per min per mg of protein (μmoles)	Percentage of inhibition
0	3.0	0	2.7	0
0.170	2.3	23	1.8	33
0.425	1.5	50	1.0	63
0.850	0.67	78	0.44	84
1.70	0.15	95	0.15	94

Effect of salt on glucose-6-phosphate dehydrogenase. The effect of NaCl on glucose-6-phosphate dehydrogenase is shown in Table 4. Although the cells had been grown in medium containing 3.75 M NaCl, we again observed significant inhibition of enzymatic activity by 0.17 M NaCl, and inhibition was complete at 1.28 M.

To test the possibility that a salt-resistant enzyme had been irreversibly denatured by preparation of the extracts in buffer without salt, cells were broken in buffer containing 3.75 M NaCl. The glucose-6-phosphate dehydrogenase activity of these extracts (Table 5) was found to be completely inhibited by 1.28 M NaCl, and no activity could be measured in the presence of a salt concentration equal to that of the medium (3.75 M).

The results obtained with cells grown in the lower salt concentration (1.28 M) are shown in

TABLE 4. *Effect of NaCl on glucose-6-phosphate dehydrogenase activity[a] in Dunaliella viridis grown in 3.75 M NaCl*

System	Activity (μmoles of NADP reduced/min)	Percentage of inhibition
Control	0.056	0
+0.17 M NaCl	0.043	23
+0.43 M NaCl	0.012	79
+0.85 M NaCl	0.003	95
+1.28 M NaCl	0	100

[a] Measured in crude extracts (0.74 mg of protein per 3 ml of assay mixture) with glycylglycine buffer.

TABLE 5. *Effect of NaCl on glucose-6-phosphate dehydrogenase activity in Dunaliella viridis extracts prepared in buffered salt solution[a]*

Concn of NaCl[b] (M)	Activity (μmoles of NADP reduced/min)
0.26	0.072
1.28	0
3.75	0

[a] The cells were grown in medium containing 3.75 M NaCl and were broken in 0.05 M Tris-chloride buffer (*p*H 7.5) containing 3.75 M NaCl. Protein content per 3-ml assay mixture was 1.1 mg.

[b] Includes NaCl added with the extract.

Table 6. The enzyme was stimulated by $MgSO_4$ and was active with NADP, but not with NAD. (These extracts had no $NADPH_2$ oxidase activity.) The salt sensitivity of the enzyme was similar to that observed with cells grown in the high salt medium (3.75 M). To explore the possibility that the inhibition observed was actually the result of an imbalance of Na^+ and K^+, rather than the effect of salt per se, the effect on the enzyme of various combinations of NaCl and KCl was tested. The results (Table 6) show that at equimolar concentrations all combinations of NaCl and KCl listed had the same inhibitory effect. When the enzyme was preincubated with salt, then diluted and assayed, the inhibition was reversed (Table 7).

Effect of salts on phosphoglucose isomerase. The NaCl sensitivity of phosphoglucose isomerase was examined and was found to be similar to that of other enzymes (Table 8). Since this enzyme does not have a metal ion requirement, it was used for testing the effect of several different cations on enzyme activity. Cesium, potassium, and lithium chlorides showed inhibition equal to that of sodium at equimolar concentrations. When tested at equal ionic strengths, $MgCl_2$ and $CaCl_2$ produced the same degree of inhibition.

Distribution of enzymatic activity. The distribution of the activity of glucose-6-phosphate dehydrogenase and phosphoglucose isomerase was studied after centrifugation of the crude extract. Both of these enzymes are generally considered to be "soluble," and, in the case of the phosphoglucose isomerase, all of the phosphoglucose isomerase activity in the *D. viridis* extracts was recovered in the supernatant fraction. However, 62% of the glucose-6-phosphate dehydrogenase activity was sedimented by centrifugation at 10,000 × *g* for 2 hr. This suggests that the enzyme is a very large molecule, or that it is attached to a

TABLE 6. *Effect of salts on glucose-6-phosphate dehydrogenase activity in Dunaliella viridis grown in 1.28 M NaCl*

System	Percentage of control activity[a]
Complete	100
− $MgSO_4$	30
− NADP, + NAD	0
+ 0.17 M NaCl	62
+ 0.43 M NaCl	35
+ 0.86 M NaCl	17
+ 1.28 M NaCl	0
+ 0.34 M NaCl	47
+ 0.34 M KCl	49
+ 0.17 M NaCl + 0.17 M KCl	47
+ 0.068 M NaCl + 0.272 M KCl	48
+ 0.272 M KCl + 0.068 M NaCl	47

[a] Activity of controls ranged between 0.05 and 0.09 μmole of substrate per min per mg of protein in crude extracts. Assays were performed in glycylglycine buffer.

TABLE 7. *Effect of preincubation with salt on glucose-6-phosphate dehydrogenase activity in Dunaliella viridis grown in 1.28 M NaCl*

System	Activity (μmoles of NADP reduced/min)
Control	0.050
+ 0.34 M NaCl	0.027
Preincubated with 0.34 M NaCl[b]	0.047

[a] Measured with a crude extract (0.915 mg of protein per 3 ml of incubation mixture) in glycylglycine buffer.

[b] Final salt concentration in assay mixture = 0.017 M. Preincubation was for 10 min in ice. Controls were preincubated under the same conditions in the absence of NaCl.

TABLE 8. *Effect of salts on phosphoglucose isomerase from Dunaliella viridis grown in 3.75 M NaCl*

Expt	Salt	Percentage of control activity[a]
1	0.3 M NaCl	57
	0.3 M CsCl	54
	0.3 M KCl	50
	0.3 M LiCl	55
2	0.3 M NaCl: $\tau/2 = 0.3$	46
	0.1 M $MgCl_2$: $\tau/2 = 0.3$	52
	0.1 M $CaCl_2$: $\tau/2 = 0.3$	50

[a] Activity in controls ranged between 0.15 and 0.20 μmole of substrate per min per mg of protein.

fragment of some cell organelle. Particle-bound glucose-6-phosphate dehydrogenase has been reported in animal cells (20, 21), but has represented only a small fraction of the nonsedimentable activity. The glucose-6-phosphate dehydrogenase of *D. viridis* seems to be quite unusual in that such a large proportion of the activity is sedimentable. Another case is that of the enzyme from *Pseudomonas fluorescens* which was found (19) to be associated with particles.

Effect of salts on enzyme activity in dialyzed extracts of D. viridis. The procedure described in the methods section for preparing crude extract involved washing the cells with salt solution. Thus, significant amounts of salt remained in the extract. For purposes of studying effects at low salt concentrations, therefore, further centrifugation and dialysis procedures were used as described in that section. (No irreversible inactivation of the enzyme occurred during dialysis; the specific activity was not reduced, provided measurements were made with the optimal salt level in the incubation mixture.) With this extract, a study of the effect of various concentrations of $MgCl_2$, NaCl, and Na_2SO_4 on glucose-6-phosphate dehydrogenase was undertaken. The cells were grown in medium containing 3.75 M NaCl. The concentration of buffer used in the assay was reduced by a factor of 10 to minimize its effects, and Tris-chloride buffer was used rather than glycylglycine, to avoid the chelating effect of the latter. Under these conditions, there was no enzyme activity in the absence of salt. The salt concentrations required to produce a given level of inhibition appear higher than in the previous experiments, as a result of the decreased ionic strength of the buffer and decreased salt concentration in the extract. Magnesium was included, since $MgSO_4$ was found to stimulate glucose-6-phosphate dehydrogenase activity in *D. viridis* (Table 6), as it does in some other organisms (2, 3, 5).

The effects of NaCl and $MgCl_2$ are compared in Fig. 2. Both salts stimulated at low concentrations and inhibited at high concentrations. When NaCl and $MgCl_2$ are compared at equal chloride concentrations (Fig. 2), it is seen that Mg^{++} both stimulates and inhibits at lower concentrations than does Na^+. (Since the molar concentration of Mg^{++} was one-half that of Na^+ at equal chloride concentrations, the effect is even more pronounced than the relative positions of the two curves would suggest.) However, when the data are plotted in terms of the ionic strengths of the salts, their effects in the stimulatory range seem to be similar, and the difference in the inhibitory effect of the two salts is lessened.

The effects of NaCl and Na_2SO_4 are compared in Fig. 3. Sodium sulfate was also found to stimulate at low concentrations (although maximal activity did not reach the level obtained with NaCl) and to inhibit at high concentrations. When the two salts are compared at equal Na^+ concentrations, both the stimulatory and inhibitory effects of $SO_4^=$ were displayed at a lower concentration of the anion than was the case with Cl^-. Again, since the salts were compared in terms of Na^+ concentration, the concentration of $SO_4^=$ was half that of Cl^-. Again, the stimulation by the two salts appears similar when the data are plotted in terms of ionic strength.

It is not possible at this time to distinguish clearly the effects of ionic strength and the effects of the individual anions and cations. It can, however, be said that the glucose-6-phosphate dehydrogenase from this halophilic alga shows the same type of response to salts as do the glucose-6-

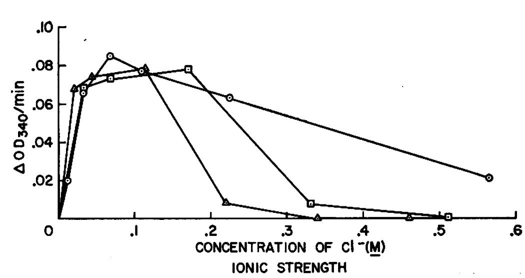

FIG. 2. *Effect of NaCl and $MgCl_2$ on glucose-6-phosphate dehydrogenase activity in dialyzed extracts of Dunaliella viridis. Symbols:* (○) *NaCl (plotted as a function of Cl^- concentration or ionic strength);* (△) *$MgCl_2$ (plotted as a function of Cl^- concentration);* (□) *$MgCl_2$ (plotted as a function of ionic strength). Incubation mixture contained: 25 μmoles of Tris-chloride buffer, pH 7.5; 2.6 μmoles of NADP; 1.0 μmole of glucose-6-phosphate; and 0.1 ml of dialyzed extract containing 0.7 mg of protein.*

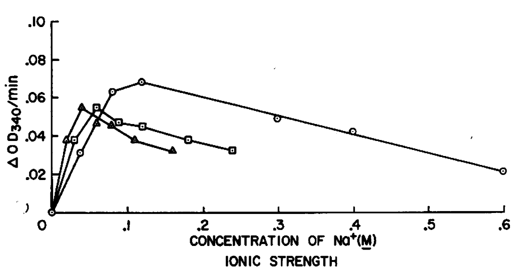

FIG. 3. *Effect of NaCl and Na_2SO_4 on glucose-6-phosphate dehydrogenase activity in Dunaliella viridis. Symbols: (○) NaCl (plotted as a function of Cl^- concentration or ionic strength); (△) Na_2SO_4 (plotted as a function of Na^+ concentration); (□) Na_2SO_4 (plotted as a function of ionic strength). Incubation mixture contained: 25 μmoles of Tris-chloride buffer, pH 7.5; 2.6 μmoles of NADP; 1.0 μmole of glucose-6-phosphate; and 0.1 ml of dialyzed extract containing 0.6 mg of protein.*

phosphate dehydrogenases from other (nonhalophilic) sources. Glaser and Brown (3) reported that the enzyme from yeast was stimulated by low levels of KCl, NaCl, $CaCl_2$, and $MgCl_2$ and inhibited by high levels of the same salts. Mangiarotti and Garrè (9) examined the effects of these ions on the yeast enzyme and concluded that the effect of NaCl and KCl actually was due to a stimulatory effect of the Cl^- combined with an inhibitory effect of Na^+ and K^+. The effect of $MgCl_2$ was reported (10) to be due to stimulatory effects by both Mg^{++} and Cl^- and an inhibitory effect of Mg^{++} at the higher concentrations. Activation of the yeast enzyme by sulfate has also been reported (11).

Effect of salts on growth. All the results reported here indicate that the enzymes from *D. viridis* are (like the enzymes from nonhalophiles) rather nonspecifically inhibited by high levels of various salts. However, the response of the whole cell to these salts is quite different, as is shown by the following experiments. Our strain of *D. viridis* showed optimal growth at salt levels between 0.8 and 2.0 M NaCl. We therefore grew cells in 1 or 1.25 M NaCl and compared the growth with that obtained when other salts were substituted for NaCl. Table 9 shows the results of an experiment in which various portions of the NaCl required for optimal growth were replaced by equimolar amounts of KCl. These results show that KCl cannot replace NaCl, and is, in fact, toxic. The results of adding low levels of KCl to a medium containing adequate levels of NaCl are also shown (Experiment 2). After 1 day of growth, inhibition was observed in the medium containing 0.10 M KCl, and inhibition was observed even at 0.05 M KCl after 2 days of growth. Levels of KCl higher than 0.1 M were extremely toxic and caused large decreases in cell number.

TABLE 9. *Effect of various salts on the growth of Dunaliella viridis*

Expt	Concn of salt	Cells/ml[a]		
		1 day	2 days	3 days
1	1.25 M NaCl	60,000		160,000
	1.00 M NaCl + 0.25 M KCl	10,000		3,000
	0.75 M NaCl + 0.50 M KCl	3,000		0
	0.50 M NaCl + 0.75 M KCl	0		0
	0.25 M NaCl + 1.00 M KCl	0		0
	1.25 M KCl	0		0
2	1.00 M NaCl	55,000	94,000	
	1.00 M NaCl + 0.05 M KCl	54,000	66,000	
	1.00 M NaCl + 0.10 M KCl	17,000	2,000	
	1.00 M NaCl + 0.20 M KCl	1,000	0	
	1.00 M NaCl + 0.30 M KCl	1,000	0	
	1.30 M NaCl	61,000	95,000	
3	1.00 M NaCl	63,000	105,000	
	1.00 M LiCl	22,000	17,000	
	1.00 M NaCl + 0.20 M LiCl	41,000	58,000	
	1.00 M NaCl + 0.40 M LiCl	36,000	43,000	
	1.40 M NaCl	59,000	94,000	
	0.67 M $MgCl_2$	21,000	10,000	
4	1.00 M NaCl	45,000	70,000	
	1.00 M $NaNO_3$	49,000	69,000	
	0.50 M Na_2SO_4	43,000	68,000	
	1.00 M CH_3COONa	15,000	9,000	

[a] Inoculum was 25,000 cells/ml.

The results expressed in Table 9 (Experiment 3) indicate that LiCl and $MgCl_2$ are also unable to replace NaCl in the growth medium. No growth was observed in 1 M LiCl or 0.67 M $MgCl_2$, and LiCl was inhibitory at 0.2 M in the presence of adequate NaCl. A study of cation requirements in another species of *Dunaliella*, the less halophilic *D. tertiolecta*, revealed that other salts can substitute for sodium as osmoregulators as long as a minimum sodium concentration (0.01 M) is maintained (14).

The effect of replacing the chloride ion with other anions is also shown (Experiment 4). The nitrate and sulfate ions supported growth at the same level as the chloride; the acetate ion did not. The chloride requirement is, therefore, not a specific one.

Measurements of freezing-point depressions on the cell sap of another closely related halophilic species, *Dunaliella salina*, have been reported (12) to indicate an internal salt concentration even greater than the high level in which these organisms were grown (3.9 M). It is difficult to reconcile this result with our finding that the enzymes of *D. viridis* are salt-sensitive.

One explanation for the ability of salt-sensitive enzymes to function in a cell having an overall high salt concentration could be the restriction of these enzymes to a cell organelle from which salt was excluded. The particulate nature of the glucose-6-phosphate dehydrogenase observed in *D. viridis* might suggest that this enzyme normally operates within a salt-free organelle which has been fragmented during preparation of the extract. However, another salt-sensitive enzyme, phosphoglucose isomerase, was found to be limited to the soluble fraction of the cell. Also, if any great part of the cell's volume were occupied by such salt-free organelles, a very high, in fact, supersaturated condition, would have to prevail in that portion of the cell from which salt was not excluded in order to yield an average salt concentration greater than 3.9 M (12). It seems unlikely, therefore, that such a mechanism is the explanation for the discrepancy between the salt sensitivity of the enzymes from *D. viridis* and the internal salt levels reported for the closely related *D. salina*.

The fact that the enzymes showed a nonspecific response to the various cations studied, whereas the whole cells had a very specific response (tolerating, and in fact requiring, high levels of Na^+, but inhibited by even low levels of other cations), suggests that the cell may possess a specific mechanism for the exclusion of sodium from the cells. Other cations would be inhibitory because the mechanism is specific for sodium, and a salt tolerance in the enzymes would not be expected because this mechanism would result in a low internal Na^+ concentration. Apparently, a similar situation occurs in another halophilic green alga, *Chlamydomonas*, in that the cells were found to have salt levels lower than those of the media (15).

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